Genisphere®

SIGNAL + SAMPLE AMPLIFICATION PRODUCTS

Biotin Labeling Kit for ST/Exon Arrays

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Introduction

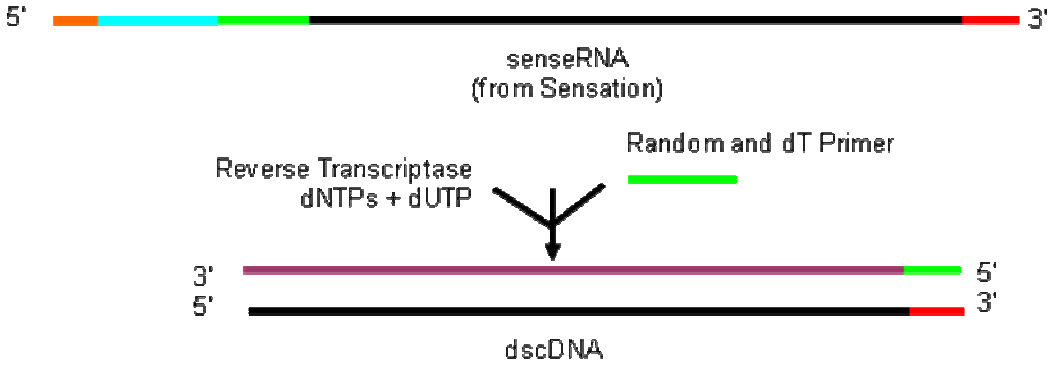
The Biotin Labeling Kit for ST/Exon Arrays contains reagents designed to convert senseRNA from Sensation kits into labeled dscDNA, for subsequent analysis on Affymetrix Whole-Transcript Expression Arrays. Optionally, unlabeled dscDNA may be analyzed by Quantitative PCR (see page 8).

Biotin Labeling for ST/Exon Arrays

Double Stranded cDNA Synthesis

3.5 hours, then 1 hour for purification

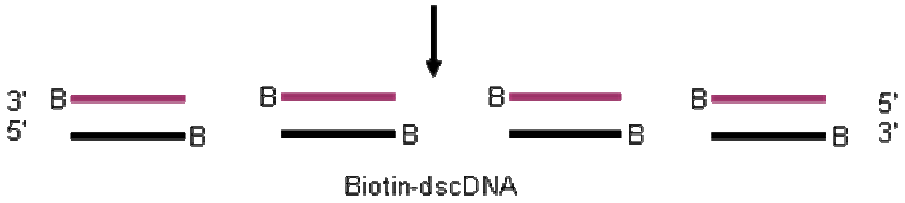
SenseRNA is reverse transcribed using random 12mer primer and dT12V primer, dNTP mix + dUTP, and Reverse Transcriptase. Second Strand cDNA is made with DNA Polymerase I and RNAse H. The dscDNA is purified with RNAClean XP.



Terminal Labeling of double stranded cDNA

1 hour

4-10ug of dscDNA is fragmented and end-labeled with 1-Step Fragment and Label Buffer and Enzyme.



Analysis of Biotin-dscDNA

The biotin-dscDNA is ready for hybridization to Affymetrix Whole-Transcript Expression Arrays.

Kit Specifications

Components and Storage

Vial 1	Labeling RT Primer Mix
Vial 2	5X MMLV RT Buffer
Vial 3	MMLV RT Enzyme
Vial 4	WT dNTP mix, 10mM dNTP + dUTP
Vial 5	1M $MgCl_2$
Vial 6	DNA Polymerase I
Vial 7	RNaseH
Vial 8	dscDNA Stop Solution
Vial 9	1M Tris-HCl, pH 7.0
Vial 10	Nuclease-Free Water
Vial 11	1-Step 5X Fragment and Label Buffer Mix
Vial 12	1-Step Fragment and Label Enzyme Mix

Store all vials at -20ºC.

Handling Kit Components

Vials 1, 2, 5, 8, 9, 10:
Thaw at room temperature, vortex, and briefly microfuge. Keep at room temperature until use.

Vials 3, 6, 7, 12:
Briefly centrifuge, then keep on ice at all times. Do not vortex.

Vials 4, 11:
Thaw on ice, briefly microfuge if necessary, and keep on ice at all times. Do not vortex.

Additional Materials/Equipment Required

for Genisphere Biotin Labeling Kit for ST/Exon Arrays

- 10-25μg senseRNA (in a volume of 23μl) from Sensation™ kits from Genisphere
- Thermal Cycler or heating devices for incubations at 4ºC, 16ºC, 25ºC, 37ºC, 42ºC, 47ºC, 65ºC, 70ºC, 75ºC, 93ºC, 99ºC
- Microcentrifuge
- Agencourt® RNAClean® XP (Beckman Coulter cat. no. A63987)
- Agencourt SPRI®Plate 96R - Ring Magnet Plate (Beckman Coulter cat. no. A29164) or equivalent magnetic plate
- 96-well round bottom microtiter plate (Costar cat. no. 3795)
- 70% Ethanol (Prepare fresh dilutions of ethanol each time RNAClean XP is used)
- 100% Ethanol
- 65ºC heat block or oven for Nuclease-Free Water (Vial 10) during RNAClean Purification
- NanoDrop™ for dscDNA quantitation (or equivalent quantitation instrument)
- Optional: materials for Gel Shift assay, Appendix A

for Affymetrix® Whole-Transcript Expression Arrays

- Hybridization oven
- Affymetrix GeneChip Command Console® Software (AGCC)
- Thermal Cycler or heating devices for incubations at 47ºC, 65ºC and 99ºC
- GeneChip® Hybridization, Wash and Stain Kit (Affymetrix cat. no. 900720)
- GeneChip Eukaryotic Hybridization Control Kit (Affymetrix cat. no. 900454)
- Fluidics station
- Scanner

Important Parameters

Procedural Notes

- 25μg senseRNA (in a volume of 23μl) is recommended for this labeling procedure. If 25μg senseRNA is not available, proceed with 10-25μg senseRNA. For each sample, use as much senseRNA as possible, up to 25μg, and not less than 10μg.

- When preparing master mixes, the enzyme should be added last and just prior to adding the master mix to the reaction. After the master mix or other reagent is added to the reaction, gently tap the bottom of the tube and briefly microfuge.

- A thermal cycler is recommended to prevent evaporation and condensation of the sample. If a thermal cycler is not available, heat blocks or water baths may be used. Microfuge the reactions after incubations, if condensation is observed.

Thermalcycler Programs

		Volume
Double stranded cDNA Synthesis		
Program 1	70ºC - 5 min, 25ºC - 5 min, 4ºC - 2 min	26μl
Program 2	25ºC - 10 min, 42ºC - 90 min, 65ºC - 10 min, hold at 4ºC for at least 2 min	40μl
Program 3	16ºC - 120 min (do not use a heated lid), 75ºC - 10 min, hold at 4ºC for at least 2 min	60μl
Program 4	65ºC - 30 min, hold at 25ºC for at least 2 min	62μl
Terminal Labeling of double stranded cDNA		
Program 5	37ºC - 60 min, 93ºC - 2 min, hold at 4ºC for at least 2 min	30μl or 60μl
Hybridization Cocktail		
Program 6	99ºC - 5 min, 47ºC - 5 min	90μl or 200μl

Procedure for Use

Double Stranded cDNA synthesis

25μg senseRNA (in a volume of 23μl) is recommended for this labeling procedure. If 25μg senseRNA is not available, proceed with 10-25μg senseRNA. For each sample, use as much senseRNA as possible, up to 25μg, and not less than 10μg.

1. Adjust the volume of senseRNA to 23μl with Nuclease-Free water (Vial 10).

2. On ice, add 3μl Labeling RT Primer Mix (Vial 1) to the 23μl senseRNA to make a 26μl senseRNA-Primer Mix.

3. Incubate the 26μl senseRNA-Primer Mix (Thermalcycler Program 1):
   70ºC for 5 minutes
   25ºC for 5 minutes
   4ºC for 2 minutes

4. For each reaction, prepare a First Strand Master Mix in a separate tube on ice:
   8μl 5X MMLV RT Buffer (Vial 2)
   3μl WT dNTP mix, 10mM dNTP + dUTP (Vial 4)
   3μl MMLV-RT Enzyme (Vial 3)
   14μl

5. Add the 14μl First Strand Master Mix to the 26μl senseRNA-Primer Mix for a volume of 40μl.

6. Incubate the 40μl reactions for first strand synthesis (Thermalcycler Program 2):
   25ºC for 10 minutes
   42ºC for 90 minutes
   65ºC for 10 minutes
   4ºC for 2 minutes

7. Prepare a fresh solution of 17.5mM $MgCl_2$ by combining 2.8μl 1M $MgCl_2$ (Vial 5) with 157.2μl Nuclease-Free Water (Vial 10).

8. For each reaction, prepare a Second Strand Master Mix in a separate tube on ice:
   12μl 17.5mM $MgCl_2$ (Vial 5 dilution from above)
   1μl Nuclease-Free Water (Vial 10)
   2μl WT dNTP mix, 10mM dNTP + dUTP (Vial 4)
   3μl DNA Polymerase I (Vial 6)
   2μl RNaseH (Vial 7)
   20μl

9. Add 20μl Second Strand Master Mix to each 40μl sample for a volume of 60μl.

10. Incubate the 60μl reactions for second strand synthesis (Thermalcycler Program 3):
    16ºC for 120 minutes (for thermalcyclers, do not use a heated lid)
    75ºC for 10 minutes
    4ºC for 2 minutes

11. Add 2μl of dscDNA Stop Solution (Vial 8) to each reaction. Proceed to page 7.

12. Incubate the 62µl samples at 65ºC for 30 minutes (Thermalcycler Program 4).

    Note: during this time, shake the Agencourt RNAClean XP Reagent bottle to resuspend the magnetic particles thay may have settled. Aliquot the appropriate amount of RNACleanXP Reagent, and keep at room temperature. For each reaction,126µl will be needed.

13. Add 8µl of 1M Tris-HCl, pH 7.0 (Vial 9) to each reaction for a volume of 70µl. Gently mix and briefly microfuge.

RNAClean XP Purification

Note: Prepare fresh dilutions of ethanol each time RNAClean XP is used.

1. If not already done, shake the RNAClean XP Reagent bottle to resuspend the magnetic particles that may have settled. Allow samples and RNAClean XP Reagent to reach room temperature.

2. Place Nuclease-Free Water (Vial 10) in a 65ºC heat block or oven.

3. For each reaction, add 126µl of RNAClean XP reagent to a well of a Costar 96 well round bottom plate. Transfer each 70µl dscDNA sample to a well containing RNAClean XP and mix well by pipetting up and down 10-20 times.

4. **<u>Add 65µl of 100% Ethanol to the dscDNA sample/RNAclean XP mixture</u> and mix well by pipetting up and down 10-20 times. Incubate for 10 minutes at room temperature (20-25°C).**

5. Place the plate onto an Agencourt SPRIPlate 96 Ring magnet (or equivalent) for 10 minutes to separate the beads from the solution.

6. Slowly aspirate and discard the cleared solution, being careful not to disturb the magnetic beads.

7. While on the magnet, add 170µl of 70% ethanol to each well and incubate for 30 seconds at room temperature. With a pipette set to 200µl, slowly aspirate the ethanol wash solution, being careful not to disturb the magnetic beads. Repeat for a total of 3 washes. **<u>Completely</u>** remove the final wash solution

8. Air-dry on the magnetic plate for 10 minutes or until all ethanol has evaporated. It may be necessary to extend the air-dry step for an additional 5 minutes until all ethanol has evaporated. Under or over-drying may result in lower sample recovery.

9. Remove the plate from the magnet.

10. Add 27µl of the 65ºC Nuclease-Free Water to each well. Once water is added to all wells, incubate at room temperature (20-25ºC) for 2-3 minutes to elute the sample from the magnetic beads. During this time, beads can be mixed by gentle shaking of the plate, or by pipetting up and down until resuspended.

11. Place the plate onto the magnet for 2-5 minutes to separate the beads from the solution.

12. Slowly aspirate the purified dscDNA sample and transfer to a new tube, being careful not to disturb the magnetic beads. Record the volume recovered.

Quantitation of dscDNA

Quantitate the dscDNA using a NanoDrop or other instrument. From the OD, determine the nucleic acid concentration of each labeled sample:

A260nm x 50 (double-stranded DNA extinction coefficient) x dilution factor
= concentration of dscDNA in ng/μl

Calculate the A260/280 ratio to determine purity. A ratio of 1.9-2.1 is desirable.

Optional: Unlabeled dscDNA may be analyzed by Quantitative PCR (do not use PCR master mixes containing Uracil N-Glycosylase).

Proceed to Terminal Labeling with 4-8μg dscDNA for 169 format arrays, or 8-16μg dscDNA for 49/64 format arrays. **For each sample, label as much dscDNA as possible in the specified range.** Store remaining dscDNA at -20ºC.

Terminal Labeling of dscDNA

Choose the procedure for Terminal Labeling based on the array format.

Terminal Labeling of dscDNA for 169 format arrays

1. Adjust the volume of 4-8µg dscDNA to 22.5µl with Nuclease-Free Water (Vial 10). For each sample, label as much dscDNA as possible, up to 8µg, and not less than 4µg.

2. For each reaction, prepare a Terminal Labeling reaction master mix in a separate tube on ice:
   6.0µl 1-Step 5X Fragment and Label Buffer Mix (Vial 11)
   1.5µl 1-Step Fragment and Label Enzyme Mix (Vial 12)
   7.5µl

3. Add the 7.5µl terminal labeling reaction master mix to the 22.5µl dscDNA for a final reaction volume of 30µl.

4. Incubate the 30µl reactions for terminal labeling as follows (Thermalcycler Program 5):
   37ºC for 60 minutes
   93ºC for 2 minutes
   4ºC for 2 minutes

5. Optional: Run the gel shift assay as indicated in Appendix A.

6. Proceed to Affymetrix Whole-Transcript Expression Array Hybridization.

Terminal Labeling of dscDNA for 49/64 format arrays

1. Adjust the volume of 8-16µg dscDNA to 45µl with Nuclease-Free Water (Vial 10). For each sample, label as much dscDNA as possible, up to 16µg, and not less than 8µg.

2. For each reaction, prepare a Terminal Labeling reaction master mix in a separate tube on ice:
   12µl 1-Step 5X Fragment and Label Buffer Mix (Vial 11)
   3µl 1-Step Fragment and Label Enzyme Mix (Vial 12)
   15µl

3. Add the 15µl terminal labeling reaction master mix to the 45µl dscDNA for a final reaction volume of 60µl.

4. Incubate the 60µl reactions for terminal labeling as follows (Thermalcycler Program 5):
   37ºC for 60 minutes
   93ºC for 2 minutes
   4ºC for 2 minutes

5. Optional: Run the gel shift assay as indicated in Appendix A.

6. Proceed to Affymetrix Whole-Transcript Expression Array Hybridization.

Affymetrix Whole-Transcript Expression Array Hybridization

Preparation of Ovens, Arrays, and Sample Registration Files

1. Turn Affymetrix Hybridization Oven on and set the temperature to **47ºC.** Set the RPM to 60. Turn the rotation on and allow the oven to preheat.

2. Mark each array package with a meaningful designation, and upload the sample and array information (sample names, barcode IDs, etc.) into AGCC.

3. Unwrap the arrays and place on the bench top, and mark each array with the corresponding designation from the wrapper. Allow the arrays to warm to room temperature.

4. Insert a pipet tip into the upper right septum to allow for venting.

Hybridization

1. Bring the reagents listed in step 3, below, to room temperature. The reagents may be found in the GeneChip Hybridization, Wash and Stain Kit, and the GeneChip Eukaryotic Hybridization Control Kit.

2. Heat the 20X Eukaryotic Hybridization Controls for 5 minutes at 65ºC.

3. For each sample, prepare a Master Mix:

	169 format	**49/64 format**
Control Oligo B2 (3nM)	1.5µl	3.3µl
20X Eukaryotic Hyb Controls	4.5µl	10µl
DMSO	**9µl**	**20µl**
Nuclease-Free Water	0µl	6.7µl
2X Hybridization Mix	45µl	100µl
Volume of Master Mix	**60µl**	**140µl**

4. Add the Master Mix (Step 3) to the biotin-labeled dscDNA to prepare the Hybridization Cocktail:

	169 format	**49/64 format**
Volume of Master Mix	60µl	140µl
Biotin-labeled dscDNA	30µl	60µl
Volume of Hybridization Cocktail	**90µl**	**200µl**

5. Incubate the Hybridization Cocktail (Thermalcycler Program 6):
   99ºC for 5 minutes
   47ºC for 5 minutes

6. Load the appropriate amount of Hybridization Cocktail onto each array:

	169 format	**49/64 format**
Volume to load on Array	90µl	200µl

7. Remove the pipet tip from the upper right septum of the array. Cover both septa with 1/2" Tough-Spots to minimize evaporation and/or prevent leaks.

8. Place the arrays into hybridization oven trays. Load the trays into the hybridization oven.

9. Hybridize with rotation at 60rpm for 16-18 hours at **47ºC**.

Washing and Staining

For additional information about washing, staining, and scanning, refer to the HWS Kit User Guide and the Affymetrix Command Console User Guide (http://www.affymetrix.com).

1. Remove the arrays from the oven. Remove the Tough-Spots from the arrays.

2. Extract the hybridization cocktail from each array and transfer it to a new tube or well of a 96-well plate in order to save the hybridization cocktail. Store on ice during the procedure, or at –80ºC for long-term storage.

3. Fill each array completely with Array Holding Buffer.

4. Allow the arrays to equilibrate to room temperature before washing and staining.

NOTE: Arrays can be stored in the Array Holding Buffer at 4ºC for up to 3 hours before proceeding with washing and staining. Equilibrate arrays to room temperature before washing and staining.

5. Place vials into sample holders on the fluidics station:
    a. Place one (amber) vial containing 600µl **Stain Cocktail 1** in sample holder 1.
    b. Place one (clear) vial containing 600µl **Stain Cocktail 2** in sample holder 2.
    c. Place one (clear) vial containing 800µl **Array Holding Buffer** in sample holder 3.

6. Wash the arrays according to array type and components used for Hybridization, Wash and Stain. For HWS kits the protocols are:

	169 format	**49/64 format**
Fluidics Protocol	FS450_0007	FS450_0001

7. Check for air bubbles. If there are air bubbles, manually fill the array with Array Holding Buffer. If there are no air bubbles, cover both septa with 3/8" Tough-Spots. Inspect the array glass surface for dust and/or other particulates and, if necessary, carefully wipe the surface with a clean lab wipe before scanning.

Scanning

The instructions for using the scanner and scanning arrays can be found in the Affymetrix Command Console Software User Manual in Chapter 6 (http://www.affymetrix.com).

References

1. Roberts L, *et al*. Identification of methods for use of formalin-fixed, paraffin-embedded tissue samples in RNA expression profiling. Genomics 2009, 94(5):341-8.

2. Pillai R, *et al*. Validation and Reproducibility of a Microarray-Based Gene Expression Test for Tumor Identification in Formalin-Fixed, Paraffin-Embedded Specimens. Journal of Molecular Diagnostics January 2011, Vol. 13, No. 1.

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Patents pending.

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Appendix A: Gel Shift Assay

Materials Required:

NeutrAvidin
4% to 20% TBE gel
Electrophoresis system with power supply
1X TBE running buffer
DNA Ladder
Gel loading dye
SYBR® Gold Nucleic Acid Gel Stain
Transilluminator

1. Dilute 1µl of each terminal labeling reaction:
   3ul Nuclease-Free water
   1µl terminal labeling reaction
   Note: 2µl of this dilution will be used for the gel shift.
   Save the remaining 2µl for repeat gel shift analysis if necessary.

2. Prepare a NeutrAvidin solution of 2 mg/mL following the manufacturer's recommendation.

3. Place a 4% to 20% TBE gel into the gel holder and load system with 1X TBE Buffer.

4. For each sample to be tested, remove two 1µl aliquots of fragmented and biotinylated sample to fresh tubes. Heat the aliquots of samples at 70ºC 2 minutes.

5. Add 5µl of 2 mg/mL NeutrAvidin to one of the two tubes for each sample tested.

6. Mix and incubate at room temperature for 5 minutes.

7. Bring the volume of DNA ladders to 6µl with water.

8. Add 4µl loading dye to all samples and DNA ladders.

9. Carefully load 10µl samples and ladders on gel.

10. Run the gel at 150 volts until the front dye almost reaches the bottom, approximately 1 hour.

11. While the gel is running, prepare 100mL of a 1X solution of SYBR Gold for staining. SYBR Gold may be diluted in 1X TBE running buffer.

12. Break open cartridge and stain the gel in 1X SYBR Gold for 30 minutes.

13. Place the gel on a UV light box and image using the appropriate filter for SYBR Gold.